

analog thereof that has been activated by an expression product of a SakK gene or functional analog thereof.

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66. The isolated nucleic acid of claim 65, wherein said repeated nucleotide sequences are selected from the group consisting of residues 7-14 and 30-38 of SEQ ID NO:6, residues 7-14 and 30-38 of SEQ ID NO:7, residues 7-14 and 30-38 of SEQ ID NO:8, residues 7-14 and 31-38 of SEQ ID NO:9, residues 7-8, 10-14 and 31-38 of SEQ ID NO:10, residues 6-7, 9-17 and 32-36 of SEQ ID NO:11, and residues 6-7, 9-17 and 32-36 of SEQ ID NO:12.

REMARKS

The Office Action of May 16, 2000 presents the examination of claims 16-34. Claims 16-43 are cancelled. Claims 44-66 are added for consideration of the Examiner. Support for the claims is found in the specification, particularly page 7, line 16 to page 8, line 21, and the original claims of the present application. No new matter is inserted into the application.

**Issues under 35 U.S.C. § 112, first paragraph**

In the Office Action dated May 16, 2000, the Examiner rejects claims 16-43 for allegedly not being described in the

instant specification. Claims 16-43 are canceled thus rendering any rejection of said claims moot. Applicants respectfully traverse any rejection under 35 U.S.C. § 112, first paragraph, as it would apply to claims 43-66. Consideration and allowance of the instant claims are requested.

*NEW MATTER REJECTION*

The Examiner states in the outstanding Office Action that, "the instant specification does not describe an inducing agent that is an expression product of a SakK and a SakR gene" and "accordingly, Applicants are required to cancel the new matter..." During an interview conducted on August 18, 2000, Applicants explained to the Examiner that the expression product of IF activates the expression product of SakK, which in turn activates the expression product of SakR, and that SakR then activates the expression of the gene of interest. In other words, SakK and SakR are not considered inducing agents.

As a result of this discussion, the Examiner requested that Applicants amend the claims to more clearly reflect the above workings of the gene expression system. In response to the Examiner's remarks, Applicants submit claims 44 and 45, as requested by the Examiner, to more clearly recite the present

invention as described on page 7, lines 16-31 of the instant specification. Applicants believe that the newly submitted claims fully clarify the meaning and scope of the present invention. Thus, the instant new matter rejection is overcome.

*FUNCTIONAL ANALOGUE*

The Examiner maintains her position that a "functional analogue" is not enabled by the specification. Applicants respectfully traverse. Reconsideration is respectfully requested.

The Examiner asserts that predictability in the art, guidance in the specification, breadth of the claims, and undue experimentation are pertinent to enablement of the present application. While this is true, Applicants disagree with the manner in which these factors were considered by the Examiner.

First, predictability in the art is adequate to produce functional analogues of the present gene expression system, as evidenced by the literature in the art. Specifically, gene expression systems analogous to the present invention, but found in other lactic acid bacteria, have been found in the art. In fact, there are several examples of functional analogues in the literature published after November 13, 1995. Examples include:

(1) Huehne et al. Microbiology, Vol. 142, pp. 1437-1448, 1996: this is a publication on the Sakacin P system; the authors did not disclose the induction phenomenon, but the paper showed that they found all of the genetic elements of the gene expression system;

(2) Nilsen et al., Journal of Bacteriology, Volume 180, pp. 1848-1854, 1998: this publication discloses a gene expression system analogous to the present system but derived from a different lactic acid bacterium;

(3) Quadri et al., Journal of Bacteriology, Vol. 179, pp. 6163-6171, 1997: this publication presents another example of an analogous gene expression system from a different lactic acid bacterium.

These publication support Applicants' claim that determination of functional analogues of the instant IF-SakK-SakR genes is well within the skill of the art. Specifically, it is well within the skilled artisan's power to engineer variants of IF, SakK and SakR that will remain biologically active. For example, functional analogues of SakR and SakK can easily be detected by sequence comparisons.

Further, an inducing peptide that is not identical to the inducing peptide mentioned specifically in the specification,

but that has the same biological activity, can be routinely produced by one skilled in the art following the teachings of the specification. Although the gene encoding the inducing peptide cannot easily be detected by sequence comparisons, there nonetheless exists a method for determining an inducing peptide. The inducing peptide is originally synthesized as a prepeptide containing a leader peptide. The leader peptide initially directs secretion of the peptide but then is cleaved off. This leader peptide can be recognized by its sequence, as shown in, for example, Quadri et al, Journal of Bacteriology, Vol. 179, pp. 6163-6171, 1997, Figure 2 ("CbnS" is the analogue of the inducing peptide). Thus, leader peptide comparison of sequences upstream from SakK and SakR homologues to identify leader peptides will lead the skilled artisan to homologous IF genes.

Second, the instant specification clearly directs the skilled artisan on how to find "functional analogues" of the inducing peptide, SakK, SakR.

*Method 1:*

1. Repeat example 1 of the instant specification. That is, find a bacteriocin-producing lactic acid bacteria that shows inducibility of bacteriocin production.

2. Subsequently, repeat example 2. That is, purify and determine the amino acid sequence of the inducing peptide, using published techniques.

3. Subsequently, clone and sequence the gene encoding the inducing peptide, using standard techniques (e.g., an oligonucleotide probe for the gene can be derived from the amino acid sequence of the inducing peptide). This procedure is not described in the specification, however, cloning a gene on the basis of a given protein sequence from an organism as simple as a lactic acid bacterium is obvious to any expert in the field. For example, the present Inventors cloned the inducing peptide gene from *L. sake* LTH673 as described in Brurberg et al., Molecular Microbiology, Vol. 26, pp.347-360, 1997, page 348.

4. Subsequently, clone and sequence DNA fragments adjacent to the gene encoding the inducing peptide, using standard genetic techniques. This will yield the complete sequence of the an "inducing peptide-SakK-SakR" operon, including its promoter, which is one possible promoter for the expression of the gene of interest. Further sequencing of adjacent sequences may yield other bacteriocin-related genes and regulatable promoters, as is the case of the Sakacin P producing lactic acid bacterium. The

identity of the genes that are discovered in this way can easily be confirmed by sequence comparisons.

An example of steps 1-3 is provided by Nilsen et al., J. Bacteriology, Vol. 180, pp. 1848-1854, 1998, wherein an inducing peptide from another lactic acid bacterium, *Enterococcus faecium*, was discovered. Further, step 4 relates strongly to method 2 as discussed see below.

Method 2 can be employed with other bacteriocin-producing lactic acid bacteria that display apparently constitutive production, and which are likely to also contain a gene regulatory mechanism analogous to the present invention. Any expert in the field could utilize method 2 based upon the instant specification. Indeed, method 2 was followed by Quadri et al, Journal of Bacteriology, Vol. 179, pp. 6163-6171, 1997, to find genes encoding functional analogues of the inducing peptide-SakK-SakR operon in another type of lactic acid bacterium (*Carnobacterium piscicola*).

Method 2:

Step 1: Isolate a bacteriocin-producing lactic acid bacterium.

Step 2: Isolate one or more of the bacteriocins and determine their amino acid sequences.

Step 3: Use the amino acid sequences to design oligonucleotide probes which are then used to clone the genes encoding these bacteriocins.

Step 4: After having cloned the bacteriocin gene(s), clone and sequence adjacent DNA fragments and analyze their sequence. Continue this process until an operon analogous to the inducing peptide-SakK-SakR or PlnABCD operon is found. Analogous operons can be recognized by their gene composition (a short gene followed by genes whose products share sequence similarity with SakK and SakR).

These elementary steps were followed by Quadri et al, Journal of Bacteriology, Vol. 179, pp. 6163-6171, 1997 and resulted in identification of an inducing peptide-SakK-SakR operon analogue. Each step represents standard biochemical and genetic methods that are well within the skill of any normally educated molecular biologist or geneticist.

The determination of functional analogues is further advanced at the end of step 3 and during step 4 by looking for promoter-like sequences showing analogy with the sequences depicted in Fig. 4 of the specification. A comparison of promoter sequences is presented in Diep et al., Journal of Bacteriology, Vol 178, pp. 4472-4483, 1996, Figure 5. Note that



the promoter elements shown in this Figure had not (and could not have) been recognized as such in literature published prior to Nov 13, 1995.

For all of the above reasons, Applicants respectfully submit that the term "functional analogue" is described and/or enabled by the instant specification. One of ordinary skill in the art could use the guidance of the instant specification to produce functional analogues of the IF-SakK-SakR gene expression system without undue experimentation. As such, Applicants respectfully request that the Examiner withdraw the instant rejection.

***Issues under 35 U.S.C. § 102(b)***

In the Office Action dated May 16, 2000, the Examiner rejects claims 16-43 for allegedly being anticipated by Diep et al. (1994) or Tichaczek et al. (1994). Claims 16-43 are canceled thus rendering any rejection of said claims moot. Applicants respectfully traverse any similar rejection, as it would apply to claims 43-66. Consideration and allowance of the instant claims are requested.

During the interview, the Examiner conceded that the present invention was novel in the fact that the inducing agent

is not a bacteriocin. Applicants consider the Examiner's remarks  
~~and submit claims with the proviso that the IF gene or the~~  
functional analogue thereof is not a lantibiotic. Applicants  
respectfully submit that the instant claims reciting said  
proviso are novel and unobvious over the cited prior art  
references, and respectfully request that the Examiner allow the  
instant claims.

*INDUCING PEPTIDE VERSUS BACTERIOGIN*

? The inducing peptide of the present invention is not a  
bacteriocin. It is well known in the art that inducing peptides  
are not the same as bacteriocins, although some of these  
peptides may exert some anti-microbial activity.

In the particular case of the plantaricin inducing peptide,  
the situation is complicated because this peptide was  
originally, but erroneously, presented in the literature as a  
bacteriocin (J. Nissen-Meyer et al., J. General Microbiology  
Vol. 139, pp. 1973-1978, 1993; see next point). It is important  
to note that on November 13, 1995, nothing was known about  
inducing compounds related to bacteriocin production. At that  
time, only bacteriocins were known.

In 1994, plantaricin A (as presented in Diep et al., 1994) was considered a bacteriocin. Only after November 13, 1995, did it gradually become clear that the primary role of plantaricin actually was that of an inducing peptide (Diep et al., 1995; for more information on this topic see Anderssen et al., Applied and Environmental Microbiology Vol 64, pp. 2269-2272, 1998).

The Examiner proposed the proviso "wherein the expression product of the IF gene is not a bacteriocin" in the instant claims because the nisin-based system is prior art. Applicants consider the Examiner's remarks and use the Examiner's suggestion, but replace "bacteriocin" with "lantibiotic". Lantibiotics are a subclass of bacteriocins to which nisin belongs. In the present invention, none of the peptides or the functional analogues thereof belong to the lantibiotics. For a formal description of the classification of bacteriocins, see T.R. Klaenhammer, FEMS Microbiology Reviews, Vol. 12, pp. 39-86, 1993.

Further, it is important to note that the nisin-system is essentially different from the present gene expression system. In nisin producing strains, nisin is both the inducing peptide and the bacteriocin. In the present invention, the inducing peptide has no or little bacteriocin-like activity. Bacteriocins

inhibit various bacterial strains at concentrations in the 1-10 ng/ml range. In contrast, inducing peptides according to the present invention have very low, if any, antimicrobial activity (much higher concentrations (> 1000-10,000 ng/ml) are needed for some antimicrobial effect).

*HISTORY; MISLEADING INFORMATION IN PRIOR ART ON THE PLANTARICIN INDUCING PEPTIDE (DIEP ET AL., 1994)*

Diep et al. (1994) contains several other errors that destroys its power as a prior art reference. First, Diep et al. shows that the PlnABCD operon is transcribed from one promoter. However, Diep et al. do not show that the promoter is regulatable, nor do Diep et al. show how it may be regulated. Most importantly, Diep et al. do not annotate the promoter sequence correctly. The putative promoters as annotated by Diep et al. are actually not the real (inducible) promoters that are used in the present invention. Correct recognition of the promoter elements is an absolute prerequisite for biotechnological exploitation of the promoters claimed in the present application.

The error of Diep et al. (1994) in annotation can be seen when comparing Fig. 1 in Diep et al. (1994) with the sequence

marked "plnA" in Figure 4 of the specification. The sequence in Fig. 4 of the specification starts at what is position 457 in Fig. 1 of Diep et al. (1994). Specifically, the true promoter, as disclosed in the present invention, is located rather far downstream of the promoters annotated by Diep et al (1994).

Applicants submit the following as evidence that Diep et al. incorrectly annotates the promoter.

First, Diep et al., Journal of Bacteriology, Vol 178, pp. 4472-4483, 1996 discloses the so-called transcriptional start site of the PlnABCD operon (Figures 4 and 5). Applicants will submit a copy of Diep et al. 1996 in a timely manner. This experimental determination of the start site also gives clues about the so-called -10 region which precedes the transcriptional start site approximately 10 nucleotides upstream. (Nb. Determination of the transcriptional start site was not discussed in Diep et al., 1994). The results in Diep et al. (1996) show that the correct -10 region (Diep et al., 1996, Fig. 5, sequence labeled with "PlnA") actually corresponds to nucleotides 527-532 in Fig. 1 of Diep et al.(1994) (TATCCT). This again corresponds with the -10 region marked in Fig. 4 of the specification. Thus, the correction made in Diep et al.

(1996) clearly shows that the sequence annotation in Diep et al. (1994) is erroneous.

Second, overwhelming evidence is published showing that the products of PlnC and PlnD and SakR bind to the repeats marked in Fig. 4 of the specification ("repeat" refers to the two boxed sequences with 20 nucleotides spacing that are shown in Figure 4 in the specification). These results are presented in P.A. Risøen et al., Molecular and General Genetics, Vol. 259, pp. 224-232, 1998 and in P.A. Risøen et al., Molecular Microbiology, Vol. 37, pp. 619-628, 2000, (Figures 2 & 3). Note again that these repeats were not marked or discussed in Diep et al. (1994); further, they were not even marked or discussed in Diep et al. (1995) because even then the presence of these repeats and their putative roles in regulatable promoters had not been recognized.

Third, binding of these gene products to the repeats is impaired by point mutations in and between the repeats. These results are presented in P.A. Risøen et al., Molecular and General Genetics, revised manuscript submitted for publication. This study clearly confirms that the repeats, not noted or suggested by Diep et al. (1994), are essential elements of the promoters. These results also show that the upstream regions

marked as putative promoters in Fig. 1 of Diep et al. (1994) are not essential elements of the promoters. This study by Risøen et al. includes experiments with promoter fragments approximately corresponding to the plnA sequence shown in Fig. 4 of the specification and not including the upstream regions annotated as possible promoters in Fig. 1 in Diep et al. (1994). Also shown in this manuscript is that binding of the products of plnC or plnD protects a region corresponding to approximately nucleotides 460 - 500 in Fig. 1 in Diep et al. (1994) from attack by nucleases. Thus, PlnC and PlnD bind to the repeats marked in Fig. 4 in the specification and not to the putative promoter regions marked in Fig. 1 in Diep et al. (1994).

Fourth and finally, *in vivo* studies with plasmids in which the plantaricin promoter was coupled to a reporter gene shown that promoter activity relies on an intact set of accurately organized repeats. Further, mutations in the repeat region were detrimental for promoter activity (P.A. Risøen et al., Molecular and General Genetics, revised manuscript submitted for publication).

Another major error committed by Diep et al. (1994) is the description of plantaricin as a bacteriocin. The plantaricin peptide was presented as a bacteriocin in early publications (J.

Nissen-Meyer et al., J. General Microbiology Vol. 139, pp. 1973-1978, 1993; this study did not contain analysis of the genes). However, after November 13, 1995, the belief that plantaricin is a bacteriocin was proven to be incorrect. The bacteriocin activity ascribed to plantaricin was presumably largely due to contaminating "real" bacteriocins that skewed the results of the experiments (see Anderssen et al., Applied and Environmental Microbiology Vol. 64, pp. 2269-2272, 1998). Thus, when the 1994 paper by Diep et al. appeared in the literature, the general idea that plantaricin was a bacteriocin still existed in the literature. Diep et al., 1994 did not in any way claim or suggest that plantaricin was not a bacteriocin.

*RESPONSE TO THE EXAMINER'S REMARKS*

On page 9, lines 3-5 of the Office Action dated May 16, 2000, the Examiner writes, "Diep et al., 1994 indeed teach that the genes of the plnABCD cluster are transcribed from a common promoter". Nevertheless, Diep et al. (1994) does not teach that this promoter is inducible by a plantaricin, nor does Diep et al. (1994) teach the correct promoter. On page 9, line 13, the Examiner writes, "The inducibility [of the promoter] is an inherent characteristic of said promoter." In general, a



promoter may be inherently inducible, but in the instant case, there is no promoter disclosed. As described in detail above, Diep et al. (1994) does not disclose the correct promoter. Diep et al. (1994) in no way discloses an essential piece of the present invention.

For all of the above reasons, Applicants respectfully submit that the present invention is neither anticipated nor obvious over the cited prior art references. Applicants respectfully request that the Examiner withdraw the instant rejection.

***Issues under 35 U.S.C. § 102(b)***

In the Office Action dated May 16, 2000, the Examiner rejected claims 16-43 for allegedly being anticipated by Diep et al. (1995). Claims 16-43 are canceled thus rendering any rejection of said claims moot. Applicants respectfully traverse any rejection, as it would apply to claims 43-66. Consideration and allowance of the instant claims are requested.

In the Interview conducted on August 18, 2000, the Examiner requested proof that the Diep et al. (1995) reference was published after the priority date of the present application. In response to the Examiners request, Applicants submit herewith

as Exhibit 1 a letter from Blackwell Science Ltd. which publishes the journal *Molecular Microbiology*, confirming the publication date. As recited in the letter, the publication date of the Diep et al. reference is December 13, 1995, whereas the submission date of the instant patent application is November 13, 1995. Therefore, the publication of the Diep et al. (1995) article did not become publicly available prior to filing of the instant patent application. Therefore, Diep et al. (1995) is not prior art and any rejection of the present claims as unpatentable over Diep et al. (1995) is overcome.

For all of the above reasons, Applicants respectfully submit that all of the present claims define patentable subject matter such that this application should be placed into condition for allowance. Early and favorable action of the merits of the present application is respectfully requested.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Kristi L. Rupert, Ph.D. (Reg. No. 45,702) at the telephone number below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

Serial No. 09/068,507

If necessary, the Commissioner is hereby authorized in this, concurrent, and further replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fee required under 37 C.F.R. 1.16 or under 37 C.F.R. 1.17; particularly, extension of time fees.

Respectfully yours,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

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Enclosure:      Exhibit 1 - Letter from Blackwell Science Ltd.